

Monoclonal Antibodies That Recognize a Polypeptide Antigenic Determinant Shared by Multiple *Caenorhabditis elegans* Sperm-specific Proteins

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Abstract. Four monoclonal antibodies that are directed against antigens present in sperm and absent from other worm tissues were characterized. Antibody TR20 is directed against the major sperm proteins, a family of small, abundant, cytoplasmic proteins that have been previously described (Klass, M. R., and D. Hirsh, 1981, *Dev. Biol.*, 84:299–312; Burke, D. J., and S. Ward, 1983, *J. Mol. Biol.*, 171:1–29). Three other antibodies, SP56, SP150, and TR11, are all directed against the same set of minor sperm polypeptides that range in size from 29 to 215 kD. More than eight different sperm polypeptides are antigenic by both immunotransfer and immunoprecipitation assays. The three antibodies are different immunoglobulin sub-

classes, yet they compete with each other for antigen binding so they are directed against the same antigenic determinant on the multiple sperm proteins. This antigenic determinant is sensitive to any of six different proteases, is insensitive to periodate oxidation or *N*-glycanase digestion, and is detectable on a polypeptide synthesized in vitro. Therefore, the antigenic determinant resides in the polypeptide chain. However, peptide fragments of the proteins are not antigenic, thus the determinant is likely to be dependent on polypeptide conformation. The antigenic determinant shared by these proteins could represent a common structural feature of importance to the localization or cellular specificity of these proteins.

MONOCLONAL antibodies directed against antigens involved in the development and motility of *Caenorhabditis elegans* spermatozoa would aid analysis of the location and function of specific gene products in both normal and mutant sperm. Many sterile mutants have been isolated that have abnormal sperm development and altered sperm motility (1, 43, 45, 46). The molecular defects in these mutants are unknown, and antibodies directed against sperm-specific antigens would help detect mislocalization of specific gene products and perhaps help identify the mutated genes. The molecular basis for motility in *C. elegans* spermatozoa is of particular interest because they are nonflagellated cells that propel themselves by crawling over a substrate, but they contain almost no actin or myosin (28). Their propulsion appears to come from the flow of membrane components newly inserted at the tip of their single large pseudopod backwards along the pseudopod surface toward the cell body (34, 35). When the pseudopod is properly attached to the substrate, this flow will propel the cell forward (28, 33). One family of abundant proteins that may participate in motility has already been shown to be localized in the spermatozoan pseudopod. These are the major sperm proteins, MSPs,¹

1. *Abbreviations used in this paper:* ABY, antibody; MSP(s), major sperm protein(s); RIA, radioimmunoassay.

which make up 15% of the sperm's total protein (5, 16, 18, 44). Monoclonal antibodies directed against the MSPs and antibodies directed against specific membrane components that participate in motility would facilitate investigation of this form of locomotion.

Antibodies have been extensively used to follow the appearance and behavior of differentiation antigens in developing organisms (3). The ability to prepare monoclonal antibodies has enhanced and expanded the study of cellular antigens of all sorts (25). Although monoclonal antibodies should be specific for one or a small number of related antigenic determinants, they have on occasion revealed antigenic determinants shared by multiple proteins, often from tissues different from the source of immunizing antigen (reviewed in references 10, 21, and 39; see also references 7, 11, 12, 19, and 32). Lane and Koprowski (21) have proposed that such shared antigenic determinants may represent common structural features underlying specific macromolecular interactions that control important biological processes.

In this paper, we describe the isolation and characterization of a monoclonal antibody directed against the MSPs as well as monoclonal antibodies directed against a polypeptide antigenic determinant shared by more than eight different minor sperm proteins. In the paper that follows (36), these antibodies

are used to follow the appearance and asymmetric segregation of their antigens during sperm development.

Materials and Methods

Nematode Strains and Culture

C. elegans strain CB1490, *him-5(el490)*, which produces fertile males at high frequency (13), was used for sperm isolation and for preparation of larvae and eggs. Strain BA17, *fem-1(hc17)* (formerly *isx-1*), grown at 25°C was used to prepare hermaphrodites that have no sperm (27). Strains were maintained on petri plates seeded with *E. coli* (4). Liquid cultures were grown, and sperm were isolated from males as previously described (28). Larvae were prepared by filtration through 10- μ m nitex nylon filters (Tetko, Elmsford, NY). Eggs were prepared by hypochlorite digestion of gravid worms.

Monoclonal Antibody Production

For monoclonal antibodies TR11 and TR20, BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were immunized by an intraperitoneal injection of 10^7 *C. elegans* sperm in complete Freund's adjuvant followed 1 mo later by a second injection in incomplete Freund's adjuvant. 3 d after the second injection, splenic lymphocytes were isolated and fused with P3X63-Ag8.653 myeloma cells (a nonsecreting variant of P3X63-Ag8 [15]) according to the methods of Lemke et al. (22). Supernatants from tissue culture wells that contained hybridomas were screened for anti-sperm antibodies by solid-phase radioimmunoassay (RIA) using 5×10^5 formaldehyde-fixed, Triton X-100-permeabilized sperm as the target antigen and 125 I-goat anti-mouse IgG as the labeled probe.

Cells that gave positive results were subcloned by limiting dilution, retested by solid phase RIA, and expanded for production of antibody. Hybridomas were also injected into pristane-primed BALB/c mice to induce ascites tumors. Antibodies were purified from ascites fluid by ammonium sulfate precipitation followed by DEAE-Affigel blue (Bio-Rad Laboratories, Richmond, CA) chromatography or by affinity chromatography on a column of goat anti-mouse Ig-coupled Sepharose according to Roux et al. (37).

The other monoclonal antibodies were prepared by a similar procedure using SP2/0 cells as the myeloma partner for fusion. Culture supernatants were first tested by both RIA and by indirect immunofluorescence microscopy on either fixed sperm or fixed worms. After cloning, culture supernatants were used as a source of antibodies for the initial characterization of these antibodies. ABY SP56 was then grown as an ascites tumor and purified as above. Difficulty was encountered growing antibody SP150 as an ascites tumor so the antibody was purified from culture supernatants by repeated fractionation on Protein A-coupled Sepharose (29). These purified antibodies were used for direct iodinations and for the competition experiments described below.

Solid Phase Antibody Assays

To determine the specificity and titer of antibodies, a simple solid phase assay was used. 96-well microtiter plates (Dynatech Corp., Baton Rouge, LA) were coated for 1 h with 10^5 sperm or an equivalent amount of protein (1.6 μ g) from other tissues. Sperm were prepared by brief sonication in phosphate-buffered saline (PBS) or PBS plus the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 5 mM *N*-ethylmaleimide. Worm tissue was prepared by sonication in a protease inhibiting solution: 50 mM glycine, pH 9.0, 1 mM EDTA, 5 mM *N*-ethylmaleimide, 2 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride. After the wells were coated with worm proteins, they were blocked with 3% bovine serum albumin (BSA), 3% hemoglobin in PBS. This solution was also used for all antibody dilutions. Dilutions of primary antibodies were added to the wells for 1 h. The amount of antibody bound was then determined by addition of 10^5 cpm/well of 125 I-rabbit anti-mouse IgG. This antibody, generously provided by Pat Gearhart (Department of Biochemistry, The Johns Hopkins School of Public Health and Hygiene), was iodinated by the Iodogen method (24) to $5\text{--}10 \times 10^6$ cpm/ μ g. Purified monoclonal antibodies were iodinated similarly. After the wells were incubated for 1 h, they were rinsed 5 \times with PBS and then cut apart and counted in a Packard Auto-Gamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). All data presented are averages of the counts of two or more duplicate microtiter wells. In preliminary experiments it was found that adding 0.1% of the detergent saponin to the assay had no effect on the counts bound so all of the antigen appears to be accessible to antibody even though it was applied to the wells as crude sonicated cells.

To determine the enzyme sensitivity of sperm antigens, the ability of sonicated sperm to block the binding of antibodies to the wells was measured.

It was found that 5×10^5 sperm/well would block 95% of the antibody binding, and standard curves with decreasing amounts of sperm were linear to 10^4 sperm/well (data not shown). To determine protease sensitivity of sperm antigens, 3×10^7 sperm were lysed by freezing and thawing three times and then solubilized in 0.1 M Tris, pH 8.0, 10 mM CaCl₂, 0.1% Triton X-100. To ensure total digestion, proteases were added at 12-h intervals, 120 μ g/addition. After 36 h at 37°C, the sample was diluted to 1 ml with PBS, boiled 10 min to inactivate the proteases, and then assayed for blocking of antibody binding. Controls of sperm incubated in parallel without proteases had the same antibody blocking activity as freshly lysed sperm.

Immunoprecipitation

Sperm were dissociated by sonication in the presence of protease inhibitors as for the solid phase assay. After addition of 1% Triton X-100, they were incubated for 2 h at room temperature and then overnight at 4°C with antibody plus rabbit anti-mouse IgG covalently coupled to Protein A-Sephadex beads (38). Beads were washed as described (9) and extracted with SDS gel sample buffer for PAGE.

Gel Electrophoresis and Immunotransfer

SDS PAGE was done as described by Laemmli and Favre (20) using 10–20% linear gradient gels. Sperm were prepared by lysing frozen cell pellets by direct addition of SDS-containing sample buffer. After electrophoresis, gels were either stained with Coomassie Brilliant Blue (8) or transferred electrophoretically to nitrocellulose (BA85, Schleicher & Schuell, Inc., Keene, NH) as described by Towbin et al. (42) except that the transfer buffer was 0.0125 M Tris, 0.096 M glycine. This lower ionic strength buffer gave better transfer in our hands. Gels were equilibrated in this transfer buffer plus 0.1% SDS for 30 min before transfer. After overnight transfer at 7 V/cm, the nitrocellulose was stained for 30 s in 0.05% Coomassie Brilliant Blue in 25% isopropanol, 10% acetic acid, destained in the same solution without dye for 5 min, rinsed with PBS, and then photographed to record the exact positions of the bands and to detect possible defects in transfer. The nitrocellulose was then blocked with 3% BSA (Armour Pharmaceutical Co., Tarrytown, NY), 3% hemoglobin (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl, 10 mM Tris pH 7.4, and 0.04% azide. The transfers could be stored at 4°C up to a month without deterioration. They were labeled with either unlabeled antibody or directly iodinated antibody for 1–3 h at 37°C in the following solution: 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.25% gelatin, 1% BSA, 1% hemoglobin, and 0.04% azide. After rinsing several times in this solution, unlabeled antibody was detected by a 1-h incubation with 2×10^5 cpm/ml of 125 I-rabbit anti-mouse IgG. Transfers were then washed for 1 h with three changes of washing solution: 1 M NaCl, 0.4% Sarcosyl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.25% gelatin, and 0.04% azide. Transfers were then autoradiographed with intensifying screens at -70°C (40). For quantitation of binding, bands were cut out of the nitrocellulose sheets after alignment with the autoradiographs, weighed, and then counted in a Packard gamma counter. Background areas were cut from the same sheet, weighed, and counted, and the appropriate cpm/g was subtracted from the cpm for each band.

Sperm proteins were fractionated on two-dimensional gels essentially as described previously (5) after dissociation by SDS and acetone precipitation. Immunotransfer and staining were as for one-dimensional gels.

In Vitro Translations

Worm RNA was prepared and translated in rabbit reticulocyte lysates (Bethesda Research Laboratories, Gaithersburg, MD) as described (5). The presence of antigen was determined by immunoprecipitation followed by SDS PAGE or by direct SDS PAGE of in vitro translations without [35 S]methionine or after storage at -70°C to allow decay of the ^{35}S followed by immunotransfer and antibody labeling.

Results

Sperm Specificity of Antibodies

Hybridoma cell lines that secrete antibodies that react with antigens present in sperm were identified and cloned. The culture supernatants of these lines or immunoglobulins purified from ascites fluid were tested for cross-reaction with other worm tissue by solid-phase immunoassay (Table I). Two antibodies, SP37 and SP97, bound to other worm tissue in

Table I. Tissue Specificity of Antibodies

Antibody	Antigen					
	None	Sperm	Eggs	Larvae	Adults	Eggs and sperm
None (BKG)	54	333	203	303	328	292
Myeloma	10	66	81	61	103	207
SP37	79	13,800	258	4,700	1,726	8,602
SP97	0	4,700	6,150	7,100	6,800	5,000
SP56	15	2,000	37	25	-31	1,500
SP150	55	16,400	36	28	4	12,000
TR11	34	22,800	88	162	77	19,500
SP144	0	700	-17	17	-7	350
TR20	18	12,800	9	53	8	11,200

The average ^{125}I cpm bound to microtiter wells is shown. Wells were coated with the antigens listed across the top and then incubated with the antibodies listed in the left column, followed by ^{125}I -anti-antibody as described in Materials and Methods. All antibodies were diluted 1:200 from culture supernatants except TR11, which was diluted from IgG purified from ascites fluid. The background counts that resulted from binding of the ^{125}I second antibody with no first antibody, shown in the first row (BKG), were subtracted from the total counts obtained for each sample to give the counts shown in the other rows. In other assays there were no counts above the myeloma control for ABY TR11 binding to larvae. For the last column, wells were coated with a mixture of 1.6 μg each of sperm and egg proteins to show that the sperm antigens could still be detected in the presence of other worm proteins.

addition to sperm and were not further characterized. Five antibodies, SP56, SP150, TR11, SP144, and TR20, bound to antigens present exclusively in sperm. One of these, ABY SP144, was not further characterized because of its low binding. Using antibodies SP150 and TR11 it was found that the solid phase assay could have detected one five-hundredth the amount of antigen present in 10^5 sperm, the amount used for the data in Table I; therefore, the antigens detected in sperm by ABYs SP150 and TR11 are less than one five-hundredth as abundant in other worm tissue, if they are present at all.

The antigens recognized by these antibodies were examined by antibody labeling of nitrocellulose transfers of sperm and worm proteins fractionated by SDS gel electrophoresis (Fig. 1). No labeling of worm and larval proteins was found for ABY SP56 and ABY TR20, which confirms their sperm specificity. ABY SP56 bound to multiple sperm protein bands that ranged in apparent size from 29 to 215 kD (designated P29 to P215). Antibodies SP150 and TR11 also labeled the same sperm protein bands (Figs. 3, 4, 6, and Table II). ABY TR20 bound to a single sperm band with identical shape and mobility to the MSPs. When ABY TR20 was used to probe two-dimensional gel transfers, it labeled all three MSP isoelectric variants (5) and no other proteins (data not shown). Thus, ABY TR20 is directed against an MSP antigenic determinant; the MSPs have already been shown to be sperm-specific (5, 17, 44).

The similar pattern of protein binding to immunotransfers by ABYs SP56, SP150, and TR11 suggested that all three antibodies might bind to the same antigenic determinant. This appears to be the case because each antibody competes with the others for binding to sperm antigens (Fig. 2). The antibodies themselves are not identical to each other, however, because their binding curves differ (Fig. 2), and because each monoclonal antibody is a different immunoglobulin subclass. By conventional immunodiffusion and immunotransfer assays with immunoglobulin class-specific anti-antibodies, ABY SP56 is IgG2a, lambda; ABY SP150 is IgG2b, kappa; ABY TR11 is IgG1, lambda (data not shown). Because of their

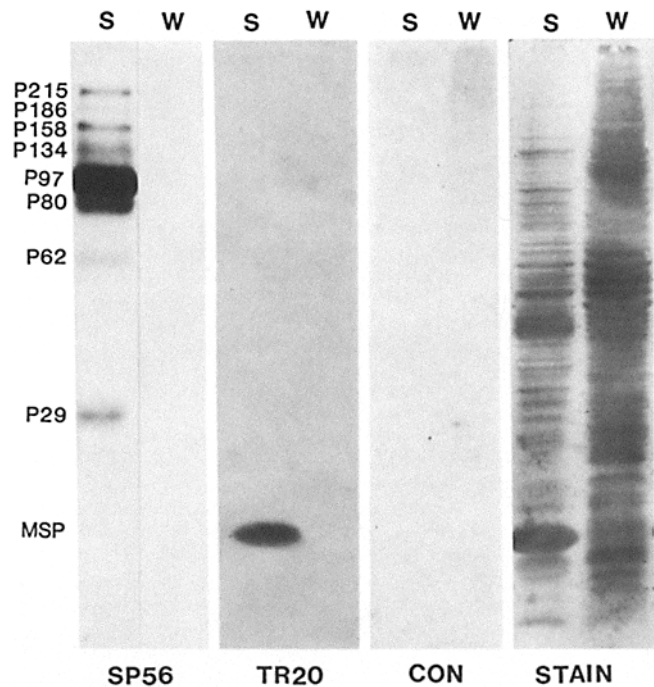


Figure 1. Antibody labeling of immunotransfers. Gel lanes were loaded with 10^7 sperm (S) or the equivalent amount of protein (160 μg) extracted from a mixture of sperm-less hermaphrodite and larval worms (W). After electrophoresis they were transferred to nitrocellulose, stained with Coomassie Blue (STAIN), and then probed with the antibodies indicated along the bottom. The antigenic sperm protein bands are labeled P (protein) followed by their apparent molecular weight in kilodaltons based on comparison to molecular weight standards or MSP. Lanes SP56 were labeled by ^{125}I -ABY SP56 at 100 ng/ml. Lanes TR20 were labeled with ABY TR20 culture supernatant 1:400 followed by ^{125}I -anti-antibody. Lanes CON were controls labeled with SP2 myeloma culture supernatant diluted 1:100, followed by ^{125}I -anti-antibody. All were autoradiographed with intensifying screens for 4–12 h. Longer exposures of the control lanes and worm protein lanes were also blank as were other controls with unrelated monoclonal antibodies.

Table II. Quantitation of Antibody Binding to Immunotransfers

Band	Percent cpm bound		
	SP150 1:200	^{125}I -ABY TR11	
		100 ng/ml	10 ng/ml
Top	—	2	2
P215	6 \pm 3	3 \pm 1	2 \pm 1
P186	4 \pm 2	3 \pm 1	3 \pm 2
P158	5 \pm 1	5 \pm 2	3 \pm 1
P131	7 \pm 4	5 \pm 1	4 \pm 2
P97	38 \pm 9	68 \pm 3	72 \pm 10
P80	16 \pm 2	8 \pm 4	7 \pm 2
P62	9 \pm 3	4 \pm 2	5
P29	10 \pm 3	1 \pm 3	6

The bands labeled in Fig. 1 and listed in column 1 were cut out of immunotransfers and counted for 20 min together with the top of the gel (Top) which occasionally trapped some antigen. Column 2 shows the mean and standard deviation of four transfers labeled indirectly with ABY SP150 culture supernatant 1:200. Columns 3 and 4 show the mean and standard deviation for three transfers labeled with ^{125}I -ABY TR11 at the concentrations indicated. The mean total cpm bound to all bands was 9,820 for ABY TR11 at 100 ng/ml, and 1,020 at 10 ng/ml, showing that the total binding was nearly proportional to antibody concentration in the range tested. Indirect labeling with ABY TR11 gave results similar to direct labeling (not shown).

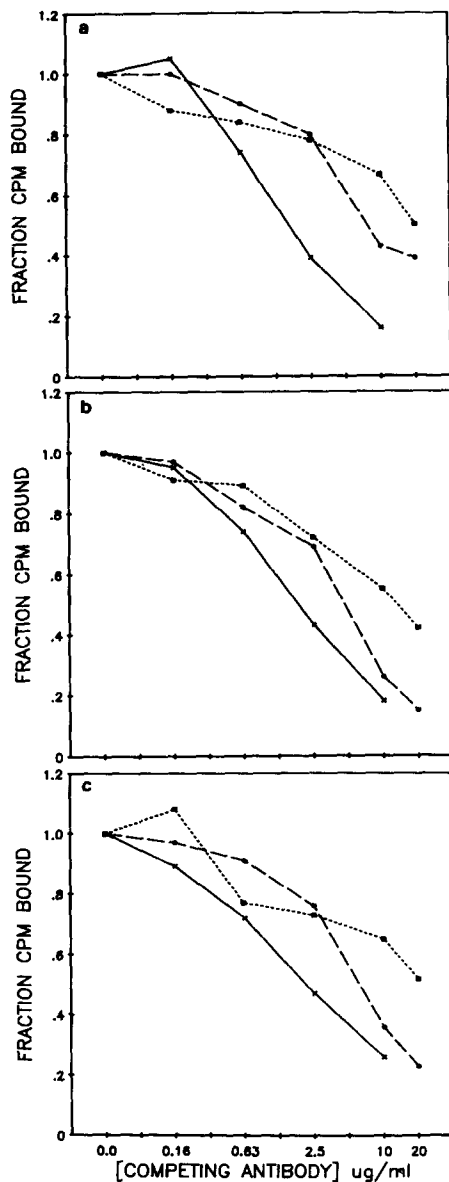


Figure 2. Antibody competition. Microtiter wells coated with 10^5 sperm were incubated with unlabeled antibodies at the concentrations indicated on the abscissa for 1 h and then ^{125}I -ABY was added to a final concentration of $0.1 \mu\text{g/ml}$. After 2 h further incubation the wells were rinsed and counted. The fraction of counts bound relative to controls with no added antibody is shown on the ordinate. All points are the average of four or more replica wells; the standard error for all points was $<10\%$ and is not shown. Controls with unrelated monoclonal antibodies showed no inhibition of binding even at higher antibody concentrations. (a) ^{125}I -ABY TR11. (b) ^{125}I -ABY SP56. (c) ^{125}I -ABY SP150. For a, b, and c, the competing unlabeled antibodies are ABY TR11, (\times); ABY SP56 (\bullet); ABY SP150 (\square).

similar antigenic specificity these three antibodies were used interchangeably for subsequent characterization of their antigens.

Multiplicity of Antigenic Polypeptides

The proportion of labeled antibody bound to each sperm protein band was quantified by cutting out the bands and determining their ^{125}I -cpm (Table II). The fraction of label in

each band was reproducible among gels, but the exact proportion of label found in each band varied among antibodies and between methods of labeling. Table II also shows that the proportion of labeling of each band did not vary significantly when the ABY TR11 concentration was reduced 10-fold even though the total amount of binding dropped ~ 10 -fold. A similar result was obtained with ABY SP150. Since the binding to each band decreased in proportion of the antibody concentration the antibodies must have similar affinities for the antigenic determinants on each of the different antigenic sperm polypeptide bands.

The apparent multiple polypeptides labeled by these antibodies could have arisen artifactually in several ways: the larger polypeptides could be aggregates of the smaller or the smaller polypeptides could be proteolytic fragments of the larger. To rule out the first possibility, each of the larger antibody binding bands was cut out of a Coomassie Blue-stained gel, refractionated on a second gel, transferred to nitrocellulose, and probed with the antibody. Each band retained its original mobility and generated no new smaller bands (Fig. 3a). The second possibility, generation of multiple bands by proteolysis, is difficult to rule out entirely, but is unlikely for several reasons. As discussed above, the multiple band pattern has been reproducible on many gels. Preincubation of sperm at 37°C for 1 h with or without solubilization by Triton X-100 before SDS PAGE and immunotransfer did not alter the band pattern significantly despite the extended opportunity for proteolysis (Fig. 3b; see also Figs. 5 and 6). Inclusions of additional protease inhibitors during sperm lysis also did not alter the band pattern (data not shown); finally,

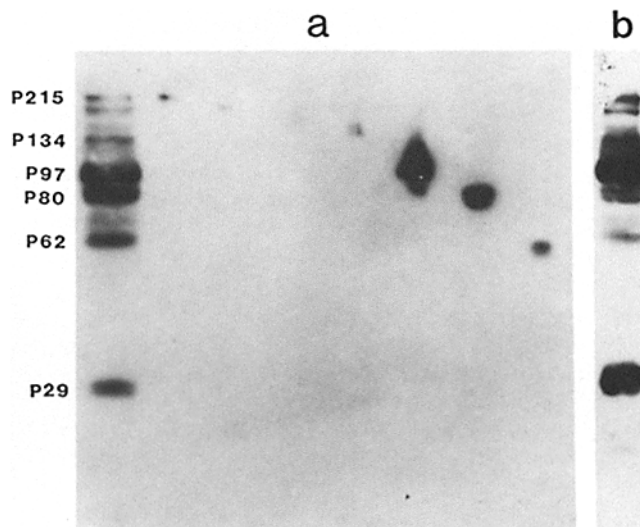


Figure 3. Reelectrophoresis of antigenic bands and the effect of sperm preincubation. (a) After SDS PAGE, a gel lane with 10^7 sperm was fixed and stained with Coomassie Blue. The antigenic bands, identified by comparison with the stained pattern on nitrocellulose and the antibody labeling pattern (Fig. 1), were cut out of the gel and then reelectrophoresed on a second gel together with a fresh lane of 10^7 sperm. This second gel was transferred to nitrocellulose and labeled with ABY SP150 followed by ^{125}I -anti-antibody. The sperm lane is on the left with bands labeled as in Fig. 1, omitting P186 and P158 for clarity. Successive lanes show the antigenic bands in order of decreasing size. (b) 10^7 sperm were solubilized in PBS plus 0.1% Triton X-100, incubated for 1 h at 37°C , acetone precipitated, fractionated by SDS PAGE, immunotransferred and probed as for a.

no protease activity has been detected in sperm homogenates (47).

To resolve the antigenic polypeptides better, sperm proteins were fractionated on two-dimensional gels, transferred to nitrocellulose, and probed with ABY SP56 (Fig. 4*b*). The major antigenic band, P97, was found to be composed of several distinct proteins, two of which were near the acidic edge of the gel. P215 and P158 were streaked bands, also acidic, whereas P186, P134, and P80 were discrete spots. On other gels P80 was a stronger spot. P29 appeared to be composed of three discrete spots and P62 was not detected. When this labeling pattern was compared with the total protein pattern of a parallel silver-stained gel (Fig. 4*a*), it was found that most of the antigenic spots were undetectable, including the two major spots corresponding to P97. Two

antigenic spots did correspond to stained spots, P80 and a minor P97 spot labeled with arrows in Fig. 4*a*. Although there are several stained spots near those corresponding to P29, careful alignment showed that they did not overlap the antigens. Two faint acidic proteins that do correspond to the major P97 antigens were found on two-dimensional gels of ³⁵S-labeled sperm proteins. These are labeled by arrows on Fig. 4*c*. One of the antigenic proteins, P80, is also the major wheat germ agglutinin binding glycoprotein in sperm (Fig. 4*d*). None of the other antigenic proteins correspond to the wheat germ agglutinin-binding proteins, however.

These two-dimensional gels show that there are more than eight protein spots that react with these sperm-specific antibodies. With the exception of P80 they are all minor sperm proteins, undetectable or barely detectable with conventional

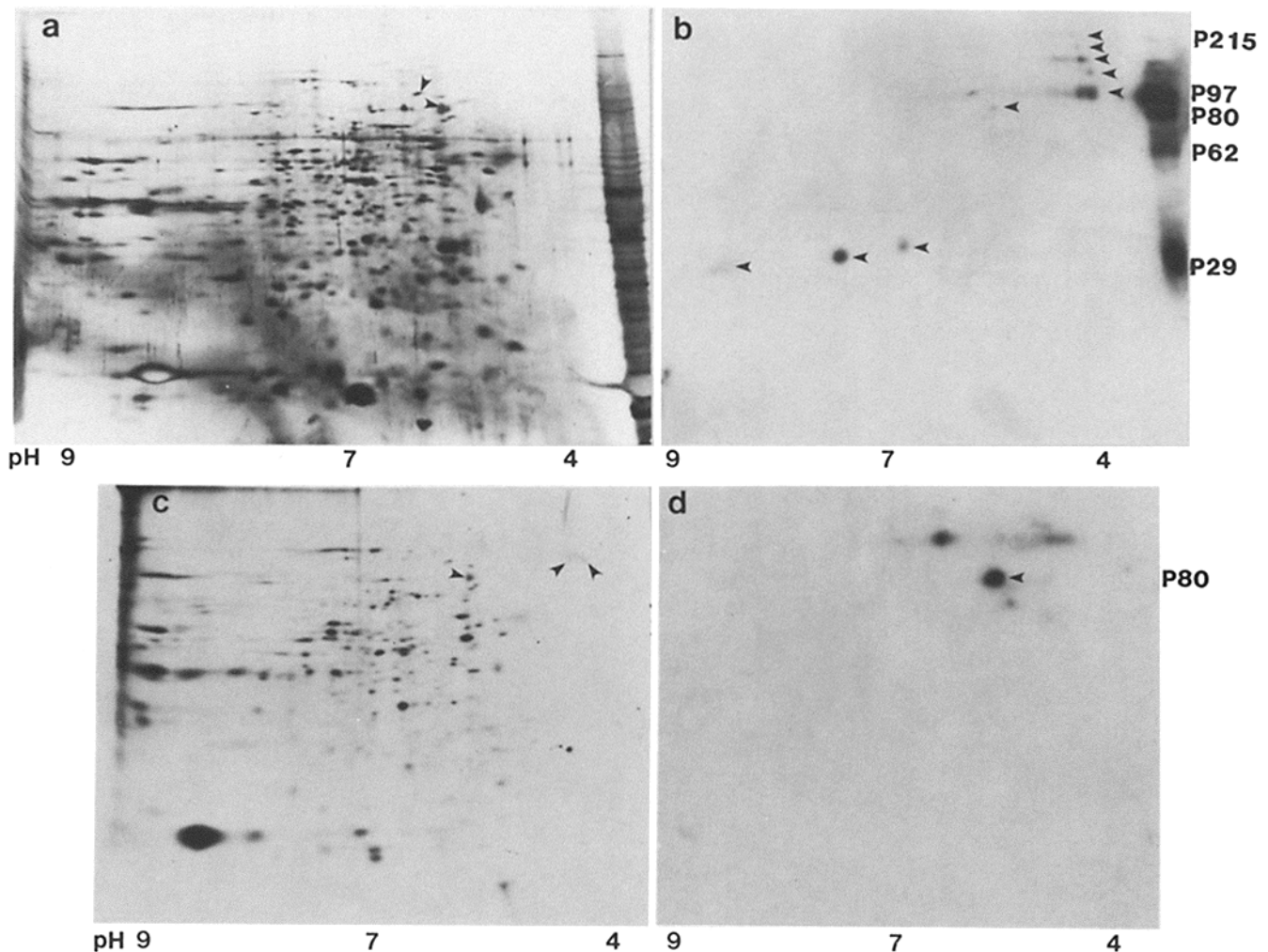


Figure 4. Two-dimensional gel fractionation of sperm proteins. Isoelectric focussing was from left to right with the pH of parallel gels indicated below. SDS PAGE was vertical. (*a* and *b*) Parallel gels of 10^7 sperm with 10^7 additional sperm on the right fractionated in the SDS gel dimension only to correlate the one-dimensional and two-dimensional gel patterns. (*a*) Silver stained; (*b*) immunotransferred and labeled with ¹²⁵I-ABY SP56. The autoradiograph was aligned using the Coomassie Blue-stained pattern of the transfer (not shown); arrows indicate the antigenic polypeptides corresponding to the one-dimensional polypeptides which are labeled along the side (P186 and P158 were omitted for clarity). Only the faint spot corresponding to P80 and the most basic spot corresponding to P97 aligned with spots detectable by silver stain and these are indicated by arrows on *a*. (*c*) Fluorograph of ³⁵S-labeled sperm proteins taken from reference 5, Fig. 11, but including the most acidic portion of the gel showing faint bands that align with the acidic spots corresponding to P97 (arrows). (*d*) Immunotransfer labeled with ¹²⁵I-wheat germ agglutinin showing that the faint antigenic spot corresponding to P80 is a major wheat germ agglutinin-binding protein, but none of the other wheat germ agglutinin-binding proteins correspond to antigenic spots.

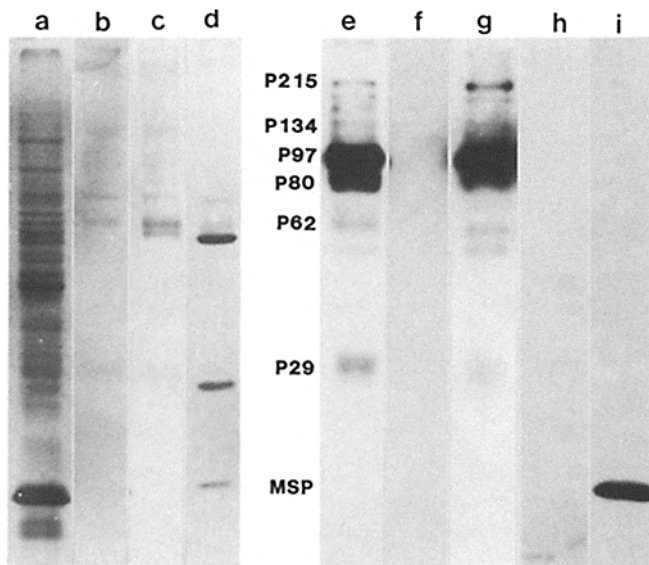


Figure 5. Immunoprecipitation of sperm antigens. Sperm were immunoprecipitated as described in Materials and Methods, and the resulting precipitated material was assayed by SDS PAGE followed by immunotransfer. (a and e) Total sperm protein; (b and f) control immunoprecipitate using rabbit anti-mouse Sephadex beads without monoclonal antibody; (c and g) immunoprecipitate using ABY TR11; (d, h, and i) immunoprecipitate using ABY TR20. (a-d) Nitrocellulose transfers stained with Coomassie Blue; (e-f) the same four transfers labeled with ^{125}I ABY SP56; (i) transfer d labeled indirectly with ABY TR20.

staining methods. The most sensitive method of detecting these proteins is immunotransfer followed by antibody labeling.

To determine the pattern of antibody binding to sperm proteins that had not first been denatured by SDS, sperm antigens were analyzed by immunoprecipitation. Sperm proteins were first solubilized with nonionic detergent, Triton X-100, and then immunoprecipitated. Since the antigens are such minor components of the sperm, the immunoprecipitates had to be assayed by SDS gel electrophoresis followed by immunotransfer and antibody labeling (Fig. 5). Staining the gel transfers of the immunoprecipitate with Coomassie Blue demonstrated that the sperm proteins detectable by stain (Fig. 5a) were not detectable in the lane immunoprecipitated with ABY TR11 (Fig. 5c). The Coomassie Blue-stained bands seen in Fig. 5c co-migrated with bands either washed off the Sephadex beads in the control (Fig. 5b) or corresponded to the immunoglobulin heavy chain. ABY TR20 immunoprecipitated an additional stained band that co-migrated with the MSPs (Fig. 5d). When these stained transfers were labeled with iodinated ABY SP56, no bands were labeled in the control or ABY TR20 lanes (Fig. 5, f and h). The ABY TR11 immunoprecipitate (Fig. 5g), however, showed the same pattern of labeling found on the direct immunotransfer of total sperm proteins (Fig. 5e), although the relative intensity of the minor bands was altered. When the ABY TR20 immunoprecipitate was relabeled with iodinated ABY TR20 the MSP band was labeled strongly, confirming its identity (Fig. 5i).

These immunoprecipitation experiments established that ABY TR11 bound to the same set of multiple native proteins

to which it bound after these proteins have been denatured by SDS and transferred to nitrocellulose. No additional proteins were detected by Coomassie Blue staining of the immunoprecipitate, but this method is relatively insensitive.

Since we wished to use these antibodies to localize their antigens in the developing sperm (36), the sensitivity of sperm antigens to the aldehyde fixatives necessary for light and electron microscopy was tested. It was found that treatment of sperm immobilized for solid-phase assay with 4% formaldehyde or 1% glutaraldehyde for 1 h did not reduce their antigenicity (data not shown) so the antigens are not sensitive to these fixatives.

The Nature of the Antigenic Determinants

An antigenic determinant shared by multiple proteins could be due to a common polypeptide structure, either a similar sequence of amino acids that form a continuous determinant, or a similar region of polypeptide folding that forms a discontinuous or conformational determinant. It is more likely, however, that an antigenic determinant shared by multiple proteins is specified by a posttranslational modification that adds the same antigenic determinant to multiple proteins. The most likely modifications to add new antigenic determinants are glycosylations (e.g., reference 10). Several experiments have been done to determine if the antigenic determinants recognized by ABYs SP56, SP150, and TR11 are specified by the polypeptide structure or by posttranslational glycosylation or other modification.

First, the sensitivity of the antigens to proteases was tested by digestion of solubilized sperm with proteases and an assay by RIA for the amount of antigen destroyed. Table III shows that the antigen was nearly completely destroyed by pronase, proteinase K, or chymotrypsin and more than half destroyed by trypsin or *Staphylococcus* V8 protease. Controls show that the proteases alone did not interfere with the assay. When samples of the digested sperm were electrophoresed on SDS PAGE only small peptides were detected by Coomassie Blue staining and no antigenic bands could be detected by immunotransfer and labeling with ABY SP150 (data not shown). Similarly, attempts to peptide map the antigens either by digesting bands cut out of gels (6) or by using sperm partially digested by Trypsin or V8 protease were unsuccessful: samples treated with enough protease to begin digesting the normal

Table III. Protease Sensitivity of SP150 Antigen

Antigen	n	Antigen fraction destroyed
Sperm alone	4	0.0 ± 0.01
Sperm + pronase	4	0.98 ± 0.0
Sperm + trypsin	3	0.5 ± 0.2
Sperm + V8 protease	2	0.6 ± 0.3
Sperm + proteinase K	2	0.98 ± 0.1
Sperm + chymotrypsin	2	0.89 ± 0.02
Proteases alone	6	0.02 ± 0.01

Sperm were digested and assayed by RIA as described in Materials and Methods. Using a standard curve prepared by adding different amounts of sonicated sperm to the wells, the fraction of antigen destroyed by each enzyme relative to a control sample without added protease (sperm alone) was calculated. The mean and standard deviation for n replicas, each with duplicate wells, is shown. Sperm were omitted from the "Proteases alone" control to show that the proteases were inactivated and did not interfere with the assay by digesting the immobilized substrate.

antigenic bands did not show any reproducible smaller antigenic peptides (data not shown). These results demonstrate that the original antigenic polypeptides were digested by all proteases, and whatever residual antigenicity was left after trypsin or V8 protease digestion could not be detected as antigenic peptide fragments. This is consistent with a conformational polypeptide determinant.

Second, attempts were made to destroy or remove potential carbohydrate antigens from sperm protein. Treatment of sperm antigens immobilized on microtiter wells or nitrocellulose with 0.2% periodate for 1 h at 4°C, which destroys many carbohydrate antigens (26), did not reduce antigenicity (data not shown). Digestion of sperm with the endoglycosidase, *N*-glycanase (peptide: *N*-glycosidase F), which should remove most asparagine-linked carbohydrates from glycoproteins (31), did not alter sperm antigens (Fig. 6, *a* and *b*). These results suggest that the antigenic determinant is not an *N*-linked carbohydrate or one sensitive to periodate.

The strongest evidence that the antigenic determinants recognized by these antibodies are polypeptides would be to show that the antigens can be detected on sperm proteins synthesized *in vitro* in a reticulocyte lysate. Posttranslational glycosylations do not occur in such lysates (23). Male worm poly A⁺-RNA was translated using a rabbit reticulocyte lysate. Antigens were not detected by immunoprecipitation of ³⁵S-labeled polypeptides, probably because they are such minor components. However, antigen synthesized *in vitro* was detected after gel electrophoresis and immunotransfer followed by iodinated antibody staining (Fig. 6, *c* and *d*). A single faint

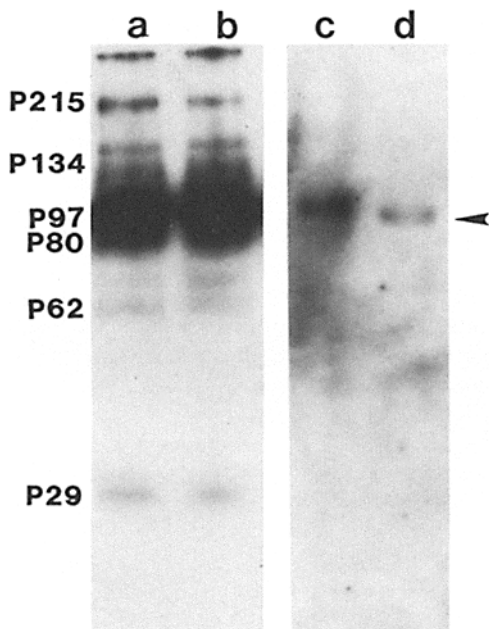


Figure 6. Treated sperm proteins and proteins translated *in vitro*. Immunotransfers of SDS PAGE gels of sperm proteins. (*a*) 10^7 sperm, no enzyme control; (*b*) 10^7 sperm digested with 1 U of *N*-glycanase (peptide: *N*-glycosidase F, Genzyme, Boston, MA) for 16 h at 30°C. (*a* and *b*) Labeled with ¹²⁵I-ABY SP56; (*c*) 2×10^5 sperm (this is 1/50 the amount loaded on other gels so the minor bands do not show up); (*d*) *in vitro* translation of 0.1 μ g of male poly-A⁺ RNA; (*c* and *d*) labeled with ¹²⁵I ABY TR11 at 10^6 cpm/ml. This is 5 \times the amount used for other gels and was necessary to increase sensitivity. Control lanes with rabbit globin mRNA showed no antigen.

antigenic band was detected with an apparent molecular weight slightly smaller than P97. Although this result was obtained twice for this particular preparation of RNA and batch of reticulocyte lysate, other RNA preparations did not produce detectable antigen. Nonetheless, the one preparation that gave positive results suggests that the antigen is present on polypeptides synthesized *in vitro*, consistent with its protease sensitivity and insensitivity to carbohydrate removal.

Discussion

Seven monoclonal antibodies that bind to sperm antigens were obtained from myeloma fusions with spleen cells from mice immunized with whole *C. elegans* sperm. Two of these, ABYs SP37 and SP97, also bind to antigens present in other worm tissue; one, ABY TR20, binds to the sperm-specific MSPs, the most abundant proteins in sperm; three, ABYs SP56, SP150, and TR11, all appear to bind to the same set of multiple minor sperm proteins; and one with low titer was not characterized further. The three antibodies that react with multiple sperm antigens do not react with nontesticular worm tissue at a level of sensitivity sufficient to detect one-five hundredth the amount of antigen present in sperm. Thus, like the MSPs recognized by ABY TR20, their antigens appear to be unique to sperm.

Antibodies SP56, SP150, and TR11, although raised independently and of different immunoglobulin subclass, all appear to bind to the same antigenic determinant which is found on more than eight sperm proteins. The evidence for this is that the three ABYs give the same pattern of protein binding on immunotransfers of sperm proteins and they compete with one another for binding to immobilized sperm antigens. Competition between two antibodies does not prove that they both bind to the same determinant; they may bind to different nearby determinants so that the binding of one antibody prevents access by the second antibody. However, because the antigenic determinants recognized by all three antibodies are shared by the same set of multiple proteins, it is most likely that these proteins all share one antigenic determinant.

The most remarkable property of these three monoclonal antibodies is that they bind to multiple sperm-specific proteins. There are a number of examples of monoclonal antibodies that bind to multiple proteins (reviewed in references 10, 21, and 39; see also references 7, 11, 12, 19, and 32). This list probably underestimates the frequency of such multiply reactive monoclonal antibodies, because many investigators would discard them early in their screening and seek antibodies specific for single proteins.

We chose to characterize these monoclonal antibodies because their antigens appear to be sperm-specific and because of the possibility that the antigenic determinants might be conserved recognition sites underlying specific macromolecular interactions involved in important biological processes (21). Before this possibility can be seriously entertained alternative explanations for multiple reactivity must first be ruled out (21). One possibility is that the same antibody molecule has recognition sites for more than one antigenic determinant. There are two arguments against this possibility: three different monoclonal antibodies all share the same pattern of binding to more than eight antigens; and the antibody dilution experiments summarized in Table II suggests that the anti-

bodies have similar affinities for the different antigenic polypeptides. It would be an extraordinary coincidence if eight different antigenic determinants all had similar affinities for an antibody molecule. It is much more likely that these eight proteins all share a single common antigenic determinant.

There remain a number of trivial ways that multiple protein bands could share a common antigenic determinant. Fig. 3 shows that the multiple antigenic protein bands are not due to noncovalent aggregation of proteins during gel electrophoresis and are unlikely to arise from artifactual proteolysis during sperm preparation. The evidence against proteolysis is that the pattern of antigenic bands is reproducible from preparation to preparation of sperm and is not altered significantly when sperm are given a prolonged opportunity for autodigestion or when additional protease inhibitors are used. It remains possible that some of the bands arise from a normal proteolysis step in sperm maturation, but there is no precedent for partial proteolysis so precisely regulated that it could give rise to a reproducible proportion of eight different antigenic polypeptides that all share the same antigenic portion of the precursor.

The most common way for multiple proteins to share a common antigenic determinant is for the antigen to be a posttranslational modification. Among cell surface differentiation antigens, the carbohydrate portions of glycoproteins are the most common antigens, and similar carbohydrates are often shared by multiple proteins (reviewed in reference 10). Noncarbohydrate posttranslational modifications can contribute to antigenicity as well (2). However, several observations suggest that the antigenic determinant recognized by ABYs SP56, SP150, and TR11 resides in the polypeptide portion of the antigenic proteins. The antigenicity is largely destroyed by any of six different proteases. Trypsin and *Staphylococcus* V8 protease seem to be less effective than the other proteases, but even short digestions of solubilized sperm or bands cut out of gels with these two enzymes do not yield antigenic peptide fragments, even from the major antigenic band P97. If the antigenic determinant were a carbohydrate or other posttranslational modification of the polypeptide, it would be expected that some of the proteases used for digestion would generate peptide fragments that retained the antigenic modification. The converse experiment, attempting to destroy the carbohydrate portion of the antigenic proteins by periodate or *N*-glycanase digestion, gave negative results: antigenicity was not lost. Although controls showed that both the periodate and the enzyme were active, neither of these agents would be expected to destroy or remove all carbohydrates on a glycoprotein. Thus, this result is consistent with but does not prove a polypeptide antigen.

Finally, with one batch of male poly A⁺-RNA we could detect an antigenic polypeptide synthesized *in vitro*. This polypeptide migrates on gels as if it were slightly smaller than the major sperm band P97, but it is not known which sperm polypeptide it represents. This result supports the evidence from the protease digestions that the antigenic determinant is in the polypeptide chains, because posttranslational glycosylations and other modifications, except N-terminal acetylation, do not occur in the *in vitro* translations (e.g., 23, 30). This antigenic polypeptide was not detected with other batches of RNA, perhaps because the antigen is such a minor component of sperm that detection requires optimal sensitivity. Slight

variations in the quality of mRNA or in the batches of reticulocyte lysate might well eliminate detectable antigen synthesis. It remains possible that N-terminal acetylation could contribute to the antigenic determinant, but the cell specificity of the antigen and its sensitivity to multiple proteases suggest that this common posttranslational modification cannot be a major antigenic determinant.

Taken together, our results suggest that the antigenic determinant recognized by these three antibodies is determined by a common polypeptide portion of more than eight antigenic proteins. Some of these proteins are modified posttranslationally: P80 is certainly a glycoprotein because it stains with periodate-silver and binds the lectin wheat germ agglutinin; P97 resolves into several spots on two-dimensional gels that differ in charge consistent with a variable modification that alters the charge, perhaps sulfation or phosphorylation. The modifications, however, are not the common antigenic determinant. They may contribute to some of the multiple protein bands, posttranslational glycosylation can alter the mobility of proteins in SDS gels (e.g., 14), but the alterations observed are not large enough to generate proteins with apparent sizes that range from 29 to 215 kD. Thus, at least some of the eight antigenic proteins must differ in their polypeptide chains.

The polypeptide antigenic determinant shared by these eight proteins appears to be a discontinuous determinant because antigenicity could not be detected on peptide fragments of the proteins. It must be a particularly robust determinant because it is found on both native proteins and proteins renatured after boiling in SDS. Recent studies of the antigenicity of peptide fragments from proteins whose three-dimensional structure is known have shown that for continuous determinants there is a strong correlation between antigenicity and conformational flexibility of the peptide region in the protein (2, 41, 48, 49). The flexibility presumably allows the peptides to adapt to conformation that binds tightly to antibody recognition site. As suggested by Tainer et al. (41), such flexible domains may well be important for protein-protein interactions in general. If so, then the most antigenic regions of a protein, which determine protein-antibody interactions, might well be the same regions that determine protein-protein interactions during cellular development. Then the antigenic determinant shared among these sperm proteins would imply similar protein interactions and presumably either shared function or similar localization.

In the following paper, it is shown by ABY SP56 immunogold labeling that its antigens are detected in several membrane and one secretory compartment during sperm development, but nearly all the antigens end up on the surface of the spermatozoa (36). The shared antigenic determinants may contribute to this localization or to the eventual function of these proteins in the spermatozoa.

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